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REVIEW ARTICLE

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The mechanism(s) of action of antioxidants: From scavenging reactive oxygen/nitrogen species to redox signaling and the generation of bioactive secondary metabolites

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Abstract

Small molecule, dietary antioxidants exert a remarkably broad range of bioactivities, and many of these can be explained by the influence of antioxidants on the redox homeostasis. Such compounds help to modulate the levels of harmful reactive oxygen/nitrogen species, and therefore participate in the regulation of various redox signaling pathways. However, upon ingestion, antioxidants usually undergo extensive metabolism that can generate a wide range of bioactive metabolites. This makes it difficult, but otherwise a need, to identify the ones responsible for the different activities of antioxidants. By better understanding their ways of action, the use of antioxidants in therapy can be improved.

This review provides a summary on the role of the *in vivo* metabolic changes and the oxidized metabolites on the mechanisms behind the bioactivity of antioxidants. A special attention is given to metabolites described as products of biomimetic oxidative chemical reactions, which can be considered as models of free radical scavenging. During such reactions a wide variety of metabolites are formed, and they can exert completely different specific bioactivities as compared to their parent antioxidants. This implies that

exploring the free radical scavenging-related metabolite fingerprint of each antioxidant molecule, collectively defined here as the scavengome, will lead to a deeper understanding of the bioactivity of these compounds.

Furthermore, this paper aims to be a working tool for systematic studies on oxidized metabolic fingerprints of antioxidants, which will certainly reveal an often-neglected segment of chemical space that is a treasury of bioactive compounds.

KEYWORDS

antioxidant mechanism of action, bioactive metabolite, free radical scavenging, oxidative stress, scavengome

1 | INTRODUCTION

The field of antioxidants undoubtedly lives its renaissance, largely due to the fact that oxidative stress is closely connected to the development of many chronic diseases, including diabetes, cancer, various aging related and central nerve system (CNS) disorders, etc.[1] Oxidative stress, a term coined by Helmut Sies,[2] has recently been redefined as *"a disruption of redox signaling and control"*,[3] emphasizing the importance of a dynamic but fine-tuned redox balance in the maintenance of cellular homeostasis.[4-7] Moreover, an intensive reconceptualization of the chemical, biological and pharmacological aspects of oxidative stress and antioxidant defense is ongoing in the field; such perspectives were recently reviewed [8-10] and will not be covered in detail here.

Small-molecule antioxidants have long been considered as compounds able to decrease or, under certain circumstances, contribute to oxidative stress. While their chemical structure makes them able to directly scavenge reactive oxygen and nitrogen species (ROS and RNS, respectively; collectively referred to as RONS),[11] the *in vivo* antioxidant action of these compounds seems to be linked to their potential to interact with various redox signaling pathways by modulation of the activity of redox enzymes.[9,10] Moreover, many dietary antioxidants undergo extensive metabolism; those that are not absorbed in the small intestines can also suffer fragmentation into smaller compounds by gut microbiota. This certainly leads to a situation where the activity observed *in vivo* is necessarily a superposition of the effects of these metabolites on several possible targets.[12,13]

This paper provides a brief summary on the various mechanisms influenced by the activity of small-molecule antioxidants. A set of dietary phenolic compounds including curcuminoids, a stilbene, hydroxycinnamic acid derivatives, a lignan, and a flavonoid are discussed in detail. These are: curcumin (**1**), demetoxycurcumin (2), bisdemetoxycurcumin (**3**), resveratrol (**4**), methyl-*p*-coumarate (**5**), methyl-caffeate (**6**), methyl-ferulate (**7**), secoisolariciresinol (**8**) and luteolin (**9**). Selection of these compounds took into consideration their chemical diversity, as well as the fact that they represent classes of natural products whose antioxidant activity attracts particularly high scientific and popular interest. The mechanisms to be discussed include free radical scavenging, modulation of antioxidant/pro-oxidant enzymes and/or transcription factors, and the formation of bioactive metabolites. Mechanisms extensively reviewed elsewhere are touched upon lightly. It is the primary aim of this paper to shed light on the potential biomedical importance of a largely neglected part of the metabolism of antioxidants, the formation of new, bioactive chemical entities upon scavenging ROS or RNS. With this idea in mind, RONS scavenging-related formation of bioactive species from ascorbic acid and estrone are also briefly discussed.

Literature search was conducted using online databases including SciFinder, PubMed, ScienceDirect and Google Scholar. Whenever possible, publications not older than 10 years were favored. However, survey on RONS

scavenging-related oxidative transformation by antioxidants was not limited to a specific time frame. This part of the search focused on biomimetic oxidative transformations of compounds **1**-**9**, and references were included only if a direct comparison between the bioactivity of the antioxidant and its oxidized analog(s) was available in the same pharmacological model.

2 | FREE RADICAL SCAVENGING AND REDOX MODULATION OF ENZYMES AND TRANSCRIPTION FACTORS

Decrease of RONS levels as a result of their scavenging by small-molecule antioxidants had been used as a simplified description of the bioactivity of these molecules, but this point of view has long been outdated. There are many easy-to-perform *in vitro* techniques available for assessing the free radical scavenging capacity of a dietary antioxidant,[11,14-16] and thanks to the simplicity of such bioassays, an abundance of related reports has been published over the last several decades. A large number of studies reported the free radical scavenging activity of the compounds selected for discussion here, and these include curcuminoids,[17] resveratrol,[18-21] cinnamic acids,[11,22,23] lignans,[24] and flavonoids.[11,25,26] Despite the popularity of such antioxidant assays, these have little if any *in vivo* relevance in terms of decreasing RONS levels. Due to the reactivity and short half-life of damaging RONS, it is now generally accepted that dietary antioxidants cannot overcome kinetic limitations in free radical scavenging to make a relevant difference in RONS levels *in vivo*.[9] A possible sole exception is the case of vitamin E that is present in large enough amounts in biological membranes to act efficiently against peroxyl radicals.[27] Nevertheless, it must be noted that, regardless of of the low efficiency of this mechanism in preventing cellular damage, RONS scavenging by dietary antioxidants (and/or their metabolites) should take place *in vivo*, and this might still deserve attention from another important perspective, namely the formation of additional metabolites, not as yet studied in detail (see Section 4).

Major ROS with large physiological/pathophysiological importance include hydrogen peroxide (H_2O_2),[28] superoxide anion radical ($O_2^{\bullet -}$),[29] hypochlorous acid (HOCl),[30] singlet oxygen ($^1O_2$),[31] hydroxyl radical ($^\bullet$OH), alkoxyl radical (RO$^\bullet$), and peroxyl radical (ROO$^\bullet$).[32] Major RNS are nitric oxide ($^\bullet$NO), nitrogen dioxide ($^\bullet NO_2$), and peroxynitrite ($ONOO^-$).[11] Primary RONS, ie H_2O_2, $O_2^{\bullet -}$, and $^\bullet$NO, have relatively low damaging potential, and their levels are under specific enzymatic control.[32,33] Structural damage of macromolecules, associated with oxidative stress and related pathologies, is connected to the more reactive and less regulated, toxic secondary species, mainly $^\bullet$OH radical, $ONOO^-$ and HOCl.[33]

It is generally well accepted that i) the production of RONS is in principle due to enzymatic processes; and ii) the primary counterbalance of the resulting oxidative stress is in fact the pool of antioxidant enzymes and not small-molecule free radical scavengers.[34] Accordingly, *in vivo* relevant antioxidant activity, ie a decrease of oxidative stress by dietary compounds, is a result of the enzymatic modulation of redox equilibrium. ROS are produced primarily by mitochondria (ETC complexes I and II), NADPH oxidases, lipoxygenases (LOXs), cyclooxygenases (COXs), xanthine oxidoreductase (XOR) and cytochrome P (CYP) monooxygenases.[32] Among RNS, the nitrogen-centered radical nitric oxide is produced by nitric oxide synthases (NOSs), and its reaction with $O_2^{\bullet -}$ leads to the formation of $ONOO^-$.[32,33] Peroxynitrite can then react with CO_2 to yield nitrosoperoxycarboxylate ($ONOOCOO^-$) that further decomposes into $^\bullet NO_2$ and carbonate radicals. Alternatively, protonation of peroxynitrite yields its acid form (ONOOH), which can undergo homolysis to yield $^\bullet NO_2$ and $^\bullet$OH radicals.[35] Though this latter reaction is less efficient than that between peroxynitrite and CO_2, it has been shown to occur and have biological significance if peroxynitrite is formed and may affect proton pumps of the cell membrane.[36]

Antioxidant enzymes counterbalance the potentially harmful effects of RONS. These can exert their function directly, such as for example superoxide dismutases (SODs), glutathione peroxidase (GPx) and catalase (CAT), or indirectly, such as for example glutathione-S-transferases (GSTs), UDP-glucuronosyl transferases, and NADP

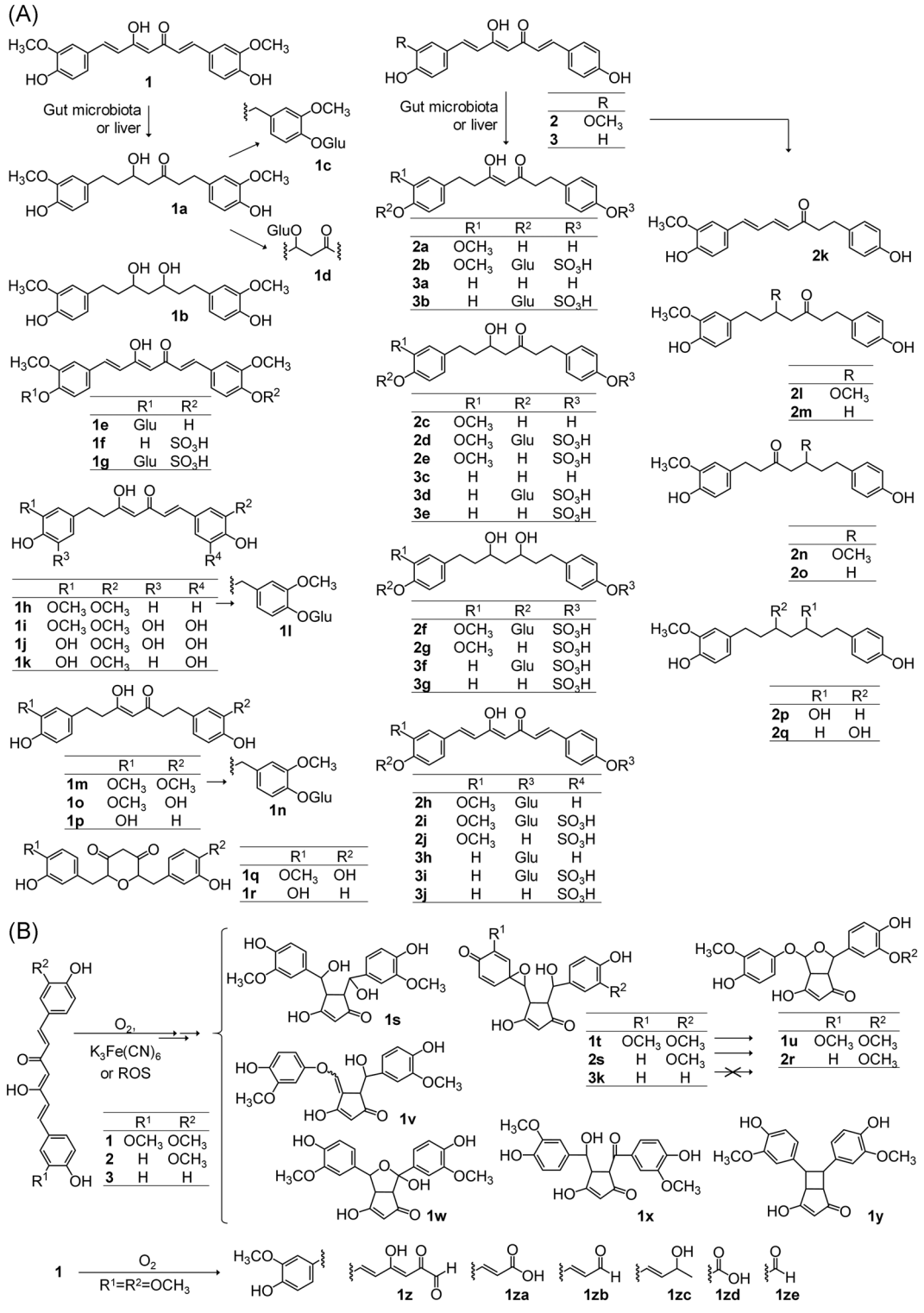


FIGURE 1 (**A**) Metabolites of curcuminoids (**1**-**3**) identified from *in vivo*, *ex vivo* and microbial fermentation studies. Bioactivity of these metabolites is discussed in Section 3.1. Glu: glucuronidyl. (**B**) Some oxidized derivatives of curcuminoids (**1**-**3**) obtained from autoxidation or biomimetic oxidative chemistry that had bioactivity profile different from that of their parent compounds, see Section 4.1 and Table 1. These compounds may likely form through free radical scavenging

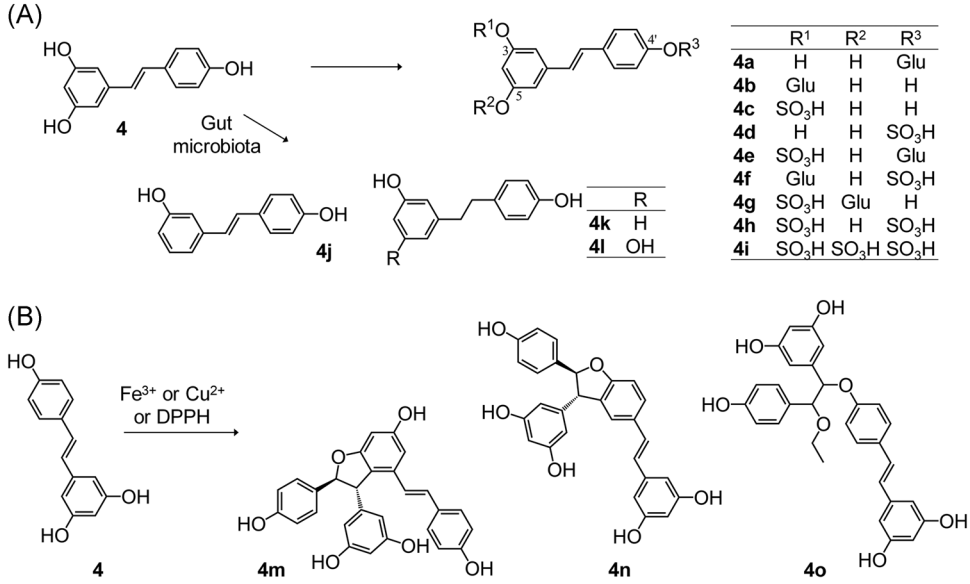


FIGURE 2 (**A**) Metabolites of resveratrol (**4**) identified from *in vitro* and *in vivo* studies. Bioactivity of these metabolites is discussed in Section 3.2. Glu: glucuronidyl. (**B**) Some oxidized derivatives of resveratrol obtained from biomimetic oxidative chemistry that had bioactivity profile different from that of their parent compound, see Section 4.2 and Table 1. These compounds may likely form through free radical scavenging

quinone oxidoreductase. The role of these enzyme systems in oxidative stress has extensively been reviewed by others.[34,37,38]

It is now also well known that certain types of ROS (eg H_2O_2) and RNS (eg $^{\bullet}NO$ and $ONOO^-$) play a pivotal signaling role. This might occur through the reversible oxidation of proteins on the SH groups of cysteine and/or methionine, and in particular, S-sulfenylation, S-glutathionylation, S-nitrosylation, S-polysulfidation or disulfide bond formation, and/or nitration of tyrosine and tryptophan residues. MAPK-mediated pathways including ERK1/2, p38 and JNK, PI3K/Akt signaling, and several transcription factors such as Nrf2, AP-1, NF-κB, HIF-1α, p53, Wnt/β-catenin, that control the expression of a large number of genes, are known to be under redox regulation. For more details on this subject and most recent developments in the field, see the review by Moldogazieva et al.[7]

Accordingly, it is not surprising that many dietary antioxidants have been reported to interfere with a variety of the above-mentioned pathways/transcription factors, and such interactions appear to play an important role in the polypharmacology of these compounds.[39] Briefly, it seems an important common feature of polyphenol compounds to induce an adaptive response to oxidative stress through the activation of Nrf2.[9] This leads to the upregulation of antioxidant enzymes including CAT, GPx, GST, paraoxonases (PONs), glutathione reductase (GR), and γ-glutamyl cysteine synthetase (γ-GCS).[39] Many plant polyphenols also share an ability to down-regulate PI3K/Akt,[40] activate AMPK [41-43] and sirtuins,[43-46] and suppress NF-κB signaling.[47] These redox-regulated pathways have also been implicated as underlying mechanisms for the health-promoting and lifespan-increasing effects of dietary polyphenols.[48]

3 | METABOLISM OF ANTIOXIDANTS IN VIEW OF THEIR BIOACTIVITY

While there is an abundance of *in vitro* pharmacological studies on dietary antioxidants, promising results obtained in such experimental systems are difficult to translate into *in vivo* applications. A major reason for this is the poor oral bioavailability of such compounds, which is largely due to their metabolism by gut microbiota and/or the

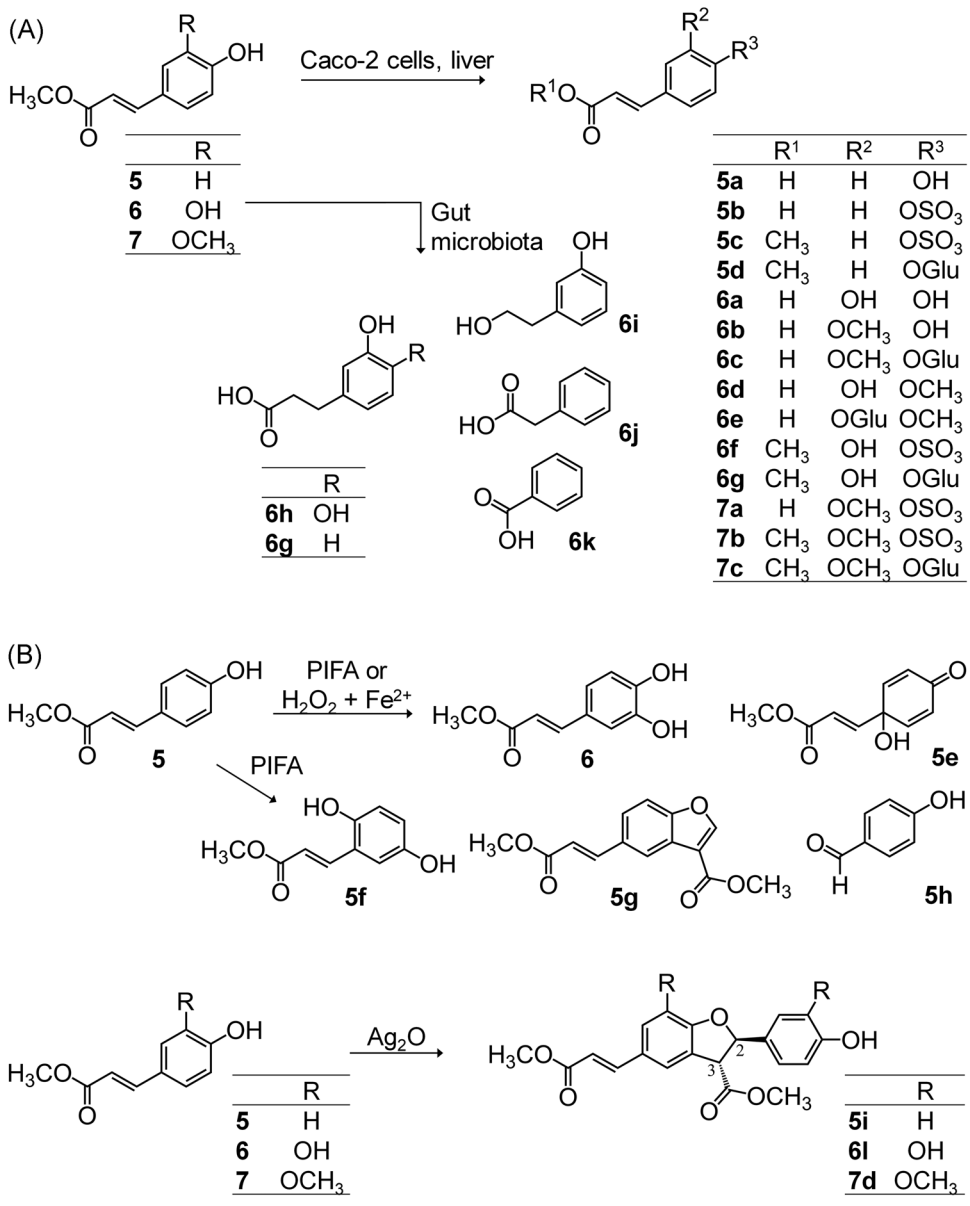


FIGURE 3 (**A**) Metabolites of methyl hydroxycinnamates (**5**-**7**) identified from *in vitro*, *in vivo* and *ex vivo* studies. Bioactivity of these metabolites is discussed in Section 3.3. Glu: glucuronidyl. Due to the methylation of **6** by catechol-*O*-methyltransferase (COMT), overlaps are observed for compounds **6** and **7**, i.e. **6b** and **7a** are metabolites of **7** and **6**, respectively. Metabolites **6h**-**6k** were obtained through cleavage of chlorogenic acid to **6a** by gut microbiota. (**B**) Some oxidized derivatives of **5**-**7** obtained from biomimetic oxidative chemistry that had bioactivity profiles different from that of their parent compounds, see Section 4.3 and Table 1. These compounds may likely form through free radical scavenging

intestinal mucosa, and the subsequent extensive metabolism of the absorbed fraction mostly within the liver and kidneys. The microbial metabolism of ingested polyphenols involves enzymatic hydrolysis of β-glycosidic bonds liberating aglycons that are absorbed better than their glycosides. Microbial metabolism frequently leads to a variety of smaller fragments, including simple phenolic acids, phloroglucinol derivatives, etc. Detailed reviews on

this subject have recently been published by others.[13,49-52] The following sub-sections attempt to provide an overview on the known Phase I and Phase II metabolic transformations of curcuminoids (**1**-**3**), resveratrol (**4**), a set of methyl-hydroxycinnamates (**5**-**7**), secoisolariciresinol (**8**), and luteolin (**9**), and on related pharmacological consequences.

To facilitate comparison between metabolites described in Sections 3 and 4, the corresponding Figures 1A-5A will present previously identified phase I and II metabolites of compounds **1**-**9**, and Figures 1B-5B metabolites obtained by biomimetic oxidative chemistry discussed in Section 4.

3.1 | Metabolism of curcuminoids

The diarylheptanoid antioxidants from Curcuma species (turmeric: *C. longa*, wild turmeric: *C. aromatica*, Javanese turmeric: *C. xanthorrhiza*), curcumin (**1**), demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**) (Figure 1), have achieved important scientific and clinical interest. At the time of this writing, 57 current clinical studies on curcumin (**1**) at various stages are registered at the National Institutes of Health (NIH) database. A wide range of *in vitro* and *in vivo* studies have claimed beneficial bioactivities attributed to curcumin (**1**), and these bioactivities include chemo-preventive, neuroprotective, antitumor and anti-mutagenic, antimetastatic, Antiangiogenic, immunomodulatory, anti-inflammatory, etc., and are extensively reviewed by others.[17,53-57]

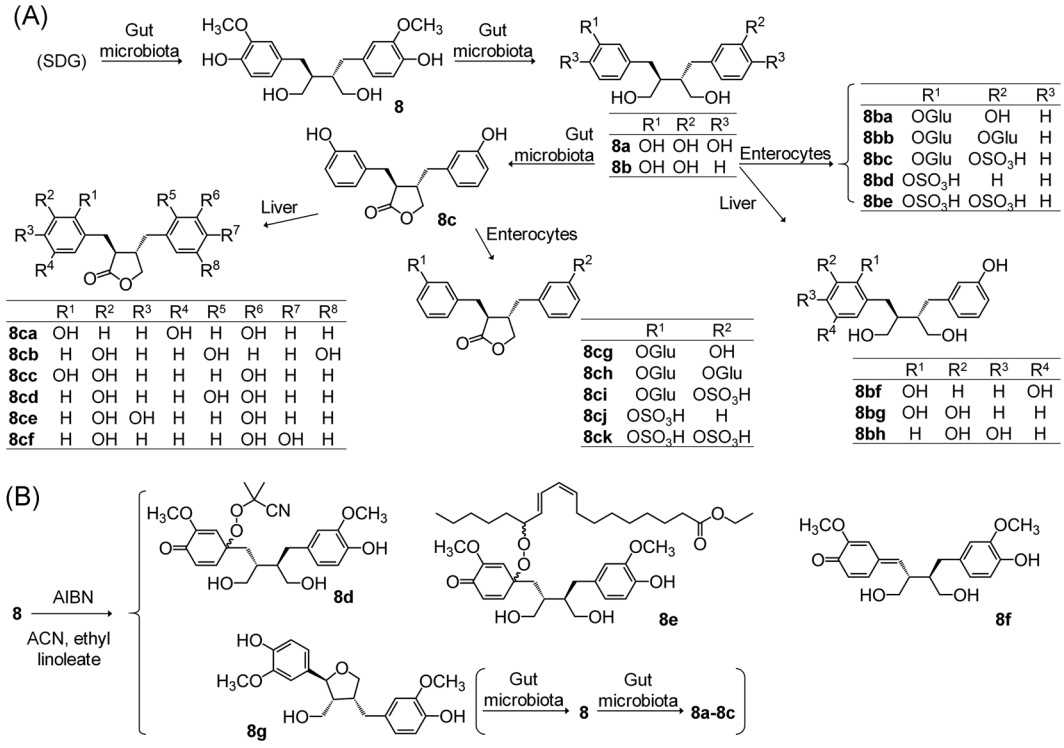
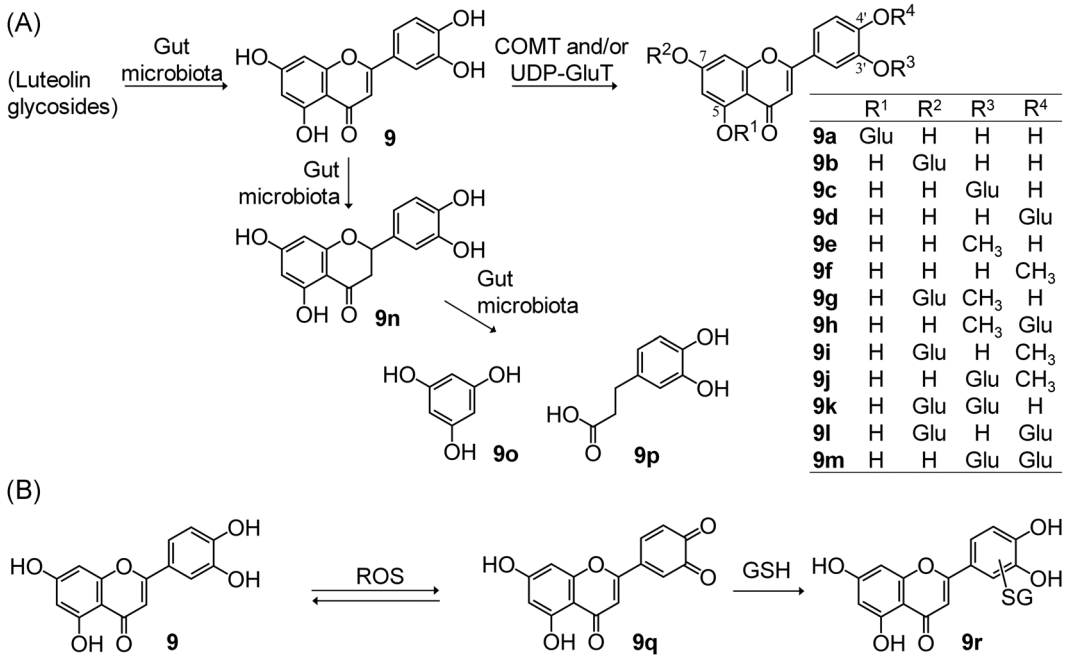


FIGURE 4 (**A**) Metabolites of secoisolariciresinol (**8**) identified from *in vitro* and *in vivo* studies. Bioactivity of these metabolites is discussed in Section 3.4. Glu: glucuronidyl, SDG: diglucoside of **8**. In case of asymmetric conjugates of **8b** and **8c**, identification was based on mass spectrometry, hence the differently substituted phenolic rings may be interchangeable.[106,107] (**B**) Some oxidized derivatives of **8** obtained from biomimetic oxidative chemistry that had bioactivity profiles that differed from that of their parent compound, see Section 4.4 and Table 1. These compounds may likely form through free radical scavenging. Lariciresinol (**8g**) is also a naturally occurring lignan that may be metabolized by gut microbiota to **8**, and subsequently to **8a**-**8c**



	R^1	R^2	R^3	R^4
9a	Glu	H	H	H
9b	H	Glu	H	H
9c	H	H	Glu	H
9d	H	H	H	Glu
9e	H	H	CH_3	H
9f	H	H	H	CH_3
9g	H	Glu	CH_3	H
9h	H	H	CH_3	Glu
9i	H	Glu	H	CH_3
9j	H	H	Glu	CH_3
9k	H	Glu	Glu	H
9l	H	Glu	H	Glu
9m	H	H	Glu	Glu

FIGURE 5 (**A**) Metabolites of luteolin (**9**) and its glycosides identified from *in vitro* and *in vivo* studies. Bioactivity of these metabolites is discussed in Section 3.5. COMT: catechol-*O*-methyltransferase, Glu: glucuronidyl, UDP-GluT: UDP-glucuronosyltransferase. The formation of phloroglucinol (**9o**) has been suggested based on the presence of dihydrocaffeic acid (**9p**). (**B**) Specific oxidized derivatives of **9** that were identified from biomimetic oxidative chemistry and are well-known to form through free radical scavenging. Glutathione-S-transferase inhibitory activity **9q** and **9r** in comparison with that of **9** is discussed in Section 4.5 and summarized in Table1

It is well understood that curcumin has very low bioavailability and low chemical stability.[58] For example, oral administration of an amount as high as 500 mg/kg of curcumin to streptozotocin-induced diabetic rats results in a maximum of only 0.06 μg/mL plasma concentration, which represents an oral bioavailability of ca. 0.5%.[59] Therefore, metabolites and decomposition products of curcumin and its analogs are thought to play a central role in their remarkable polypharmacology.[56,60,61]

Structures of the known metabolites of curcuminoids (**1**-**3**) identified from *in vivo* (mice, rats, or humans) and *ex vivo* (tissue slices) studies, as well as those from fermentation with gut microbiota, are presented in Figure 1A as compounds **1a**-**1r**, **2a**-**2r**, and **3a**-**3k**. The biotransformation of curcuminoids is known to involve reduction (a major metabolic step by gut microbiota [62,63] and by the liver [64,65]), demethylation and/or further hydroxylation, cyclization (by gut microbiota [62]), and the formation of glucuronide and/or sulfate conjugates primarily at phenolic OH groups (accordingly, **1d** is a minor metabolite).[64-68] Sulfate conjugation of curcumin (**1**) was recently also demonstrated to take place in breast cancer cells, and rapid excretion of its conjugates took place through a yet unidentified efflux transporter, possibly ABCG2.[69] As previously mentioned, free, unchanged curcumin appears to be scarcely present in the peripheral blood and tissues after oral ingestion.[66] When administered intravenously in mice, it has a half-life of around 30 minutes.[70] The main hepatic metabolite of curcumin (**1**) in humans and rats was found to be hexahydrocurcumin (**1a**) that can further be reduced to octahydrocurcumin, also referred to as curcuminol (**1b**).[64] Moreover, at least in rats, phase I metabolism can involve further reduction and methoxylation of the heptyl chain (forming **2l** and **2n**), as evidenced by the isolation of demethoxycurcumin metabolites (**2k**-**2q**) from rat feces and urine.[71]

The reduction and/or conjugation certainly influences the bioactivity of curcumin. In terms of affecting COX-2 expression, these metabolic changes may represent bio-inactivation.[64] The reduced metabolites tetra-, hexa-, and

octahydrocurcumin (**1m**, **1a**, and **1b**, respectively) are also less active than curcumin as inhibitors of lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) and NF-κB activation in RAW 264.7 macrophages.[72] However, Lee et al found that hexahydrocurcumin (**1a**) exerts potent anti-inflammatory activity by inhibiting LPS-induced, COX-2 derived prostaglandin E_2 production in RAW264.7 cells with an IC_{50} value of 0.7 μM.[73] Although in this study curcumin (**1**) was unfortunately not used as a control, Mohd Aluwi et al found it to act with an IC_{50} value of ca. 16 μM.[74] Therefore, it seems that hexahydrocurcumin (**1a**) is more potent in this regard than its parent compound, although no direct comparison was made in this case. A broad range of further bioactivities of hexahydrocurcumin (**1a**) shows that this compound is a potent bioactive metabolite of curcumin (**1**) and this has been recently reviewed by Huang et al.[75] A number of studies demonstrated that the *in vitro* antioxidant activity of **1a** is at least comparable to that of curcumin as scavenger of DPPH, •NO, and •OH radicals, and as inhibitor of AAPH-induced linoleic oxidation and hemolysis.[76-78] Hexahydrocurcumin (**1a**) was also reported as a potent antioxidant *in vivo* such that a single intraperitoneal injection of 10 to 40 mg/kg dose significantly decreased the MDA and •NO levels and the expression of NF-κB and COX-2 in a rat stroke model. This same study found **1a** to protect the rats' brain from ischaemia/reperfusion injury, and to reduce inflammatory response.[79] Curcumin was also studied and found effective in a similar rat model, however at a much larger dose (300 mg/kg).[80] Unfortunately, this makes it difficult to draw any comparison between the efficacies of the two compounds. Nevertheless, there is no doubt of the importance of the activity of metabolite **1a** *in vivo*. Most recently, this compound was also found to efficiently inhibit corneal neovascularization, an important reason for corneal blindness, induced by the p-bFGF-SAINT-18 & p-VEGF-SAINT-18 complex in an *in vivo* rat model, and it exerted ca. 50% inhibition after 6 days at a dose of 1 μg.[81]

3.2 | Metabolism of resveratrol

Resveratrol (**4**; Figure 2) is probably the most popular dietary antioxidant. This stilbene is best known as the "magic" constituent of blue grape skin and red wine, and it occurs in lower amounts in several foods including a variety of berries (eg cranberry, mulberry), tomato skin, peanuts, pistachios, cocoa, etc.[82] Resveratrol (**4**) offers a broad spectrum of health benefits including its widely accepted cardiovascular protective effect and its ability to reduce cancer risk, even though the related literature is very diverse and not fully conclusive as to its real value.[83]

The pharmacokinetics and metabolism of resveratrol (**4**) has most recently been reviewed by Wang & Sang.[84] Despite its rapid absorption, the extensive and fast metabolism makes the bioavailability of resveratrol extremely low. Oral ingestion of as high as 5 g of resveratrol by humans was found to lead to peak plasma concentrations of ca. 2.4 μM, while resveratrol-3-*O*-sulfate (**4c**; Figure 2A) could reach 8 times higher concentration and over 20 times higher AUC value.[85] Major metabolic transformations of resveratrol involve phase II and include glucuronide and/or sulfate conjugation, leading to metabolites **4a** to **4i**, and reduction by gut microbiota to form compounds **4j** to **4l**.[84] Conjugation takes place in the intestinal mucosa and liver, and the sulfate conjugate **4c** was also identified to form in adipocytes.[86] Relative importance of the different conjugation routes shows dose-dependency. Administration of smaller, 5 to 50 mg doses of resveratrol (**4**) led to the appearance of glucuronides as main metabolites in the plasma of human volunteers. In contrast, when larger, ≥ 250 mg doses were administered, the sulfate conjugate **4c** was observed as the major metabolite.[84]

Conjugation significantly alters the bioactivity profile of resveratrol in a way that some activities decrease or disappear, while others are at least partially retained.[87,88] The Na^+ salts of compounds **4i** and **4h** are much weaker inhibitors of NF-κB, COX-1, and COX-2, as well as of the •NO production of LPS-stimulated RAW 264.7 macrophages as compared to resveratrol. However, the Na^+ salt of compound **4c** and the K^+ salt of compound **4d** showed COX-1 inhibition (IC_{50} = 3.60 and 7.53 μM, respectively) comparable to that of their parent compound (IC_{50} = 6.65 μM). Both of these metabolites exerted a similarly strong inhibition of COX-2, i.e. they lost the selectivity of resveratrol towards COX-2 that is about 10 times (IC_{50} = 0.75 μM).[88] As an interesting change in bioactivity due to metabolism, compound **4c** was found to exert a selective antiestrogen effect on estrogen

receptor α in a transformed yeast model and also in MCF-7 cells. Resveratrol acted as a partial agonist in this study.[89] Resveratrol 3-glucuronide (**4b**) and 3-sulfate (**4c**) conjugates also play an important role in tissue accumulation. Following a 6 weeks-long intraperitoneal daily treatment of streptozoticin-induced diabetic rats with 5 mg/kg of resveratrol (**4**), conjugates **4b** and **4c** were found to accumulate in the heart but not in the pancreas tissue. Importantly, concentrations of **4c** (reaching a maximum of 20 nM) positively correlated with the improvement of cardiac function and haemodynamic performance.[90] It is worth noting that since deconjugation can also take place, the circulating conjugates can as well be considered as a pool of resveratrol to release in certain tissues.[83,84]

3.3 | Metabolism of hydroxycinnamates

Hydroxycinnamic acids are among the most abundant dietary antioxidants, present in many vegetables, fruits, cereals and beverages.[91] These compounds can occur in plants either in free form, or as conjugates (eg as esters in chlorogenic acids, rosmarinic acid, etc.), and they are also well known as structural elements in the biosynthesis of many plant phenolic compounds such as lignans or flavonoids.[23,91-93]

After oral ingestion, enteral metabolism of methyl esters of *p*-coumaric, caffeic and ferulic acids (compounds **5**, **6**, and **7**, respectively; Figure 3) involves de-esterification [94] and primarily sulfate or, to a lesser extent, glucuronide conjugation, leading to the formation of compounds **5a**-**5d**, **6a**-**6g**, and **7a**-**7c**, respectively (Figure 3A).[95] In case of the catechol methyl caffeate (**6**) and its metabolite caffeic acid (**6a**), methylation by catechol-*O*-methyltransferase (COMT) also takes place, with subsequent formation of the sulfate conjugates of ferulic acid (**6b**) and isoferulic acid (**6d**) as main products.[94-96] Following absorption, glucuronidation, methylation and/or sulfation takes place in the liver and the formed metabolites participate in enterohepatic circulation. Methylation by COMT was also observed in the kidneys.[92] Extrapolating from results obtained for chlorogenic acid (5-*O*-caffeoylquinic acid), it is also clear that bacterial metabolism of caffeic acid involves side-chain saturation as a major step (forming **6h**), and that it can also be dehydroxylated and undergo gradual side-chain shortening all the way down to benzoic acid (forming **6g** and **6i**-**6k**).[97,98] Similar metabolic transformations are expected for compounds **5** and **7**, as well.

Unsurprisingly, the bioactivity profiles of methylated, glucuronidated and sulphated metabolites of hydroxycinnamic acids show significant differences as compared with those of their parent compounds.[92] The *in vitro* antioxidant activities such as ferric reducing activity and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, as well as the antibacterial activity of the methylated and/or conjugated metabolites typically decrease as compared to their corresponding parent compounds. However, some activities are at least partially retained. Heleno et al found that the acyl glucuronidated derivatives of compound **5a** and those of its expectable metabolite *p*-hydroxybenzoic acid exert stronger cytotoxicity on some human cancer cell lines than their parent antioxidant.[99] It should be noted that these compounds were of synthetic origin and the metabolism-related formation of acyl glucuronides has not been reported for compounds **5**-**7** nor for their demethylated forms.

3.4 | Metabolism of secoisolariciresinol

Secoisolariciresinol (**8**; Figure 4) is a lignan present in largest amounts in its diglycoside (SDG) form in flax (*Linum usitatissimum* L.) seed, and it is widely acknowledged for its health benefits in preventing many lifestyle-associated problems, including cardiovascular disease and metabolic syndrome, and it also appears to have chemo-preventive activity against cancer, particularly colorectal cancer.[100-102]

Secoisolariciresinol (**8**) is a classic example for a compound where transformation by gut microbiota is crucial for the formation of major bioactive metabolites, so called mammalian lignans or enterolignans, particularly enterodiol (**8b**) and enterolactone (**8c**); for chemical structures, see Figure 4A. These compounds are efficiently absorbed in humans [103] and seem to play a significant role in the health-preserving effect attributed to flax seed consumption.[104] In healthy postmenopausal women, 86 or 172 mg single oral dose of an extract containing 43%

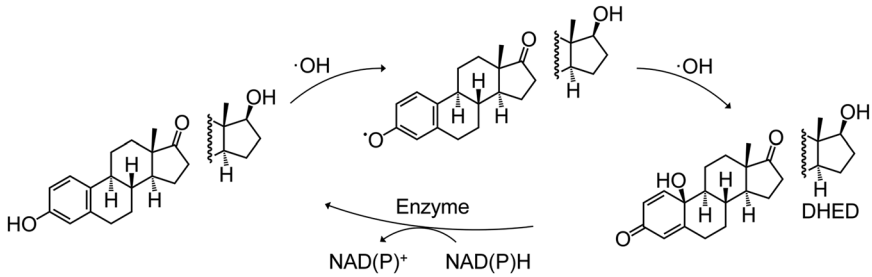


FIGURE 6 Cyclic antioxidant mechanism for estrone and estradiol, involving a chemically stable, *p*-quinol intermediate formed through •OH radical scavenging. DHED was successfully applied *in vivo* as a brain-targeted neuroprotective pro-drug of estradiol[147-149]

SDG (ie 37 or 74 mg SDG) resulted in peak plasma concentrations of ca. 250 or 540 ng/mL (**8**), 35 or 75 (**8b**) and 40 or 50 ng/mL (**8c**).[103] Subsequent to bacterial deglycosilation, demethylation, and dehydroxylation of SDG (and lactone ring formation in case of **8c**),[105] **8b** and **8c** undergo phase II metabolism during absorption by colon epithelial cells to form sulfate and glucuronide conjugates (**8ba**-**8be** and **8cg**-**8ck**).[106,107] The free, nonconjugated fractions of both **4b** and **4c** undergo microsomal oxidative metabolism in the liver, leading to hydroxylated products at the aromatic rings (**8bf**-**8bh** and **8ca**-**8cf**) or in the aliphatic region.[108,109] Structures of the latter metabolites, however, have not been unequivocally identified, hence they are not presented here.

The enterolignans **8b** and **8c** are well-known for their bioactivity.[110] Briefly, they are antioxidants themselves, and act as phytoestrogens both at the receptor level and through competing with endogenous steroid hormones for plasma protein binding. In addition, **8c** also inhibits key enzymes of the human steroid metabolism including aromatase,[111] 5α-reductase,[112] cholesterol-7α-hydroxylase [110] and 17β-hydroxysteroid dehydrogenase.[113] Epidemiological evidence suggests the preventive role of **8b** and **8c** in breast and prostate cancer,[110] and several recent *in vitro* and *in vivo* studies imply their positive effect in a variety of cancers including ovarian,[114] colorectal,[115] and lung cancer.[116] However, while **8b** and **8c** have extensively been studied, little is known on the bioactivity of their further metabolites.

3.5 | Metabolism of luteolin

Luteolin (**9**; Figure 5) is a catechol-type B-ring containing flavone, present in many fruits, vegetables and medicinal plants, and valued for a wide range of health benefits including anti-inflammatory, neuro-protective and chemo-preventive effects as detailed in the reviews by Kwon [117] and Aziz et al,[118] and the recent book by Dwight.[119]

In healthy human subjects, a single-dose oral intake of 20 mg/kg of luteolin resulted in a peak plasma concentration of ca. 330 ng/mL at 1 hour after administration,[120] suggesting that bioactivities observed *in vitro* at around 0.1-1 μM concentrations might have *in vivo* relevance. As to its metabolic fate, several recent studies addressed the metabolism of luteolin (**9**) in cell cultures and/or after oral ingestion in rodents.[121-123] Luteolin (**9**) is absorbed faster in the small than in the large intestine, and undergoes a significant intestinal first-pass metabolism through conjugation to mono- and diglucuronides (**9a**-**9d**, **9k**-**9m**) by UDP-glucuronosyltransferases,[121] and, in parallel to this, *O*-methylation by COMT forms chrysoeriol (**9e**) and diosmetin (**9 f**) that are then glucuronidated as is the parent compound [123] (chemical structures are presented in Figure 5A). Administering a single 20 μM/kg oral dose in rats, tissue distribution of luteolin (**9**) and its metabolites **9b**-**9g**, **9i** and **9j** was found to be rapid into the organs, with the exception of **9e** and **9j** whose presence was not detectable in the brain at any time probably due to the blood-brain barrier. In most tissues, the 3′-glucuronide conjugate (**9c**) was the predominant metabolite reaching peak concentrations of *ca.* 2.5 nM/g in the small intestine, *ca.* 0.7 nM/g in the lungs, and *ca.* 0.5 nM/g in the stomach, liver and kidney, and the peak plasma concentration was *ca.* 1.5 nM/mL. In contrast, luteolin (**9**) itself reached only a

0.6 nM/mL peak plasma concentration in that study, and its concentration in the organs was also typically below that of **9c**.[124] Unlike luteolin, its glycosides reach the large intestine where the sugar moieties can efficiently be cleaved by gut bacteria. Subsequent bacterial metabolism of luteolin involves the reduction of the 2,3-double bond to form the flavanone eriodictyol (**9n**) whose cleavage to phloroglucinol (**9o**) and 3-(3,4-dihydroxyphenyl)propionic acid (**9p**) can also take place.[125]

Concerning the bioactivity of luteolin metabolites, the anti-inflammatory activity of three major mono-glucuronide conjugates (**9b**-**9d**) was recently studied in comparison with that of their parent compound in LPS-treated RAW264.7 cells. At 25 μM, luteolin (**9**) could completely prevent the LPS-induced increase in the mRNA expression of *IL-6*, *IL-1β*, *NF-κB1*, *Ccl2*, *Ccl3* and *Ccl5*. While the main metabolite **9c** was inactive at the same concentration, **9a** and particularly **9b** were found to partially retain the activity of their parent compound, with **9b** acting ca. half as strong as luteolin (**9**).[126] Considering the concentrations that can be achieved *in vivo*, compounds acting weak at 25 μM concentration might seem to have low chance to exert a relevant *in vivo* activity. However, evidence suggests that a microenvironment-dependent deconjugation can also take place: an unspecified mono-glucuronide of **9**, most likely **9c**, was found to be hydrolyzed to free luteolin by human β-glucuronidase released by neutrophils.[127] This can largely influence the bioactivity of circulating luteolin conjugates during inflammation processes.[128]

4 | BIOLOGICAL SIGNIFICANCE OF RONS SCAVENGING-RELATED ANTIOXIDANT METABOLITES

The structure and function of antioxidants changes upon RONS scavenging. It needs to be stressed that the sensitive chemical structures of these compounds make them subjects not only to reversible redox circles, but their oxidation can clearly result in chemically stable metabolites as well. Moreover, this can take place not only due to well-controlled enzymatic oxidation for example through the CYP450 system, but it is also possible through RONS scavenging. For example, the relatively stable RNS peroxynitrite ($ONOO^-$; first half-life at pH = 7.2 is ca. 2.2 seconds [129]) can not only oxidize but also hydroxylate and nitrate phenolic rings [130,131] leading to stable products that cannot be changed back into their parent compounds through a simple reduction. While the scavenging itself has little chance to significantly decrease RONS levels, the forming oxidized species need to be considered as part of the locally-forming metabolite-fingerprint in the biological compartment where this takes place.

Studies reviewed in this Section suggest that due to their altered 3D chemical structure as compared to that of the parent antioxidant, it is reasonable that any such metabolites have the potential to interfere with other signaling pathways, enzymes, receptors or cell machinery, i.e. their ROS/RNS-dependent formation will have an impact on the observed bioactivity. Moreover, the ensuing bioactivity must not be limited to redox processes and related signaling pathways, but any druggable target has the chance of being involved. It is also worth noting that since the commonly observed polypharmacology of antioxidants is a result of a superposition of a wide variety of mechanisms including those affected by the bioactive metabolites, any stable, individual metabolite should likely act in a more specific way than its parent compound. The below-discussed vitamin C and estrone provide good examples to demonstrate the biological relevance of this phenomenon.

It is of particular interest that the oxidized form of vitamin C, dehydroascorbic acid (DHA) was identified as a specific inhibitor of IκBα Kinase β (IKKβ) and IKKα, regulators of the transcription factor NF-κB.[132] NF-κB plays an important and complex role in stress, immune response, cell death and survival, and ROS have an activating effect on IKKβ.[133] As such, vitamin C plays a dual role in modulating these pathways, both pointing in the same direction. On the one hand, ascorbic acid acts as an antioxidant decreasing ROS levels (hence decreasing their potential to activate IKKβ), while DHA, formed through ROS scavenging, specifically inhibits the above-mentioned kinase.[132] The activation pathways of NF-κB, including the IKK kinases, are considered as emerging antitumor targets,[134] but the very high number of pro- or antiapoptotic genes [135] controlled by NF-κB makes the overall picture complex.

TABLE 1 Comparison of the bioactivities of antioxidants (AO) **1**-**9** and their oxidized metabolites (OM) obtained from biomimetic oxidative chemistry. Chk1/Chk2: Checkpoint kinases 1 and 2, CR: cross-resistance, dox: doxorubicin, $L5178_{B1}$: L5178 cells transfected with the human ABCB1 transporter, $NCI\text{-}H460_{Dox}$: NCI-H460 cells adapted to dox, Topo II: topoisomerase II, XO: xanthine oxidase.

				Bioactivity of	
A-O	Oxidizing agent	OM	Bioactivity target	AO	OM
1	$K_3Fe(CN)_6$	Reactive intermediates	Topo II	Inactive at 50 μM[164]	Relative DNA cleavage at 5 μM 3-fold (Topo IIα) or 2-fold (IIβ)[164]
	O_2 (autoxidation)	**1z**, **1za**, **1zb**, **1zc**, **1ze**	XO	Experimental: conflicting[170,171] *In silico*: K_d = 141 (keto) or 186 μM (enol)[166]	*In silico*: **1z**, **1za**, **1zb**, **1zc**, and **1ze**: K_d = 4.57, 70.8, 24.0, 91.2, and 97.7 μM, respectively[170]
	O_2 (autoxidation)	**1z**, **1za**, **1zb**, **1zd**, **1ze**	CYP450 enzymes	CYP1A2, CYP3A4, CYP2D6, CYP2C9, CYP2B6 IC_{50} = 40.0, 16.3, 50.3, 4.3, and 24.5 μM[169]	**1zb** CYP1A2 and CYP2B6: IC_{50} = 228 and 260 μM, all others > 300 μM[169]
	O_2 (autoxidation)	Reactive intermediates	NF-κB	IC_{50} = 18 μM; depends on autoxidation rate[167]	Stable oxidation products less potent; intermediates covalently bind to IKKβ at Cys179[167]
2	$K_3Fe(CN)_6$	Reactive intermediates	Topo IIα	Inactive at 25 μM, 2-fold relative DNA cleavage at 50 μM[165]	2-Fold relative DNA cleavage at 25 μM[165]
3	$K_3Fe(CN)_6$			Inactive at 100 μM[165]	1.5-Fold relative DNA cleavage at 10 μM[165]
4	$FeCl_3$	Mixture at 33% conversion	Hydroperoxidase activity of LOX	Inactive at 100 μM[180]	98% Inh. at 50 μM[180]
	$FeCl_3$ or $CuCl_2$	**4m**			IC_{50} = 17 μM[180]
	$FeCl_3$ or DPPH	**4n**			IC_{50} = 62 μM[180]
5	PIFA	**5f**	Cancer cell viability	Very weak on a diverse cell line panel; strong CR with Dox; L5178 IC_{50} = 125 μM	Weak CR with Dox; L5178, $L5178_{B1}$ IC_{50} = 5 and 12 μM; NCI-H460, $NCI\text{-}H460_{Dox}$ IC_{50} = 3.8 and 7 μM[183]
	PIFA or $FeSO_4 + H_2O_2$	**5e**		$L5178_{B1}$ IC_{50} > 150 μM NCI-H460 IC_{50} = 120 μM $NCI\text{-}H460_{Dox}$ IC_{50} = 1043 μM[183]	No CR with Dox; L5178, $L5178_{B1}$ IC_{50} = 0.46 and 0.62 μM; NCI-H460, $NCI\text{-}H460_{Dox}$ IC_{50} = 1.85 and 2.06 μM[183]

(Continues)

TABLE 1 (Continued)

A-O	Oxidizing agent	OM	Bioactivity target	Bioactivity of AO	Bioactivity of OM
	PIFA or $FeSO_4 + H_2O_2$	**5e**	Chk1/Chk2	Inactive at 10 μM[183]	Chk1-S345p↓, Chk2-T68p↑ at 10 μM[183]
6	Ag_2O	**6l**	Cancer cell viability	Contradictory on MCF-7: IC_{50} ca. 100 μM[184] or 0.62 μM[185]	MCF-7: IC_{50} = 29 nM; MDA-MB-435, MDA-N, BT-549 IC_{50} < 10 nM[186]
	Ag_2O	**6l**	Angiogenesis	Inactive in zebrafish[190]	(*2 R,3 R*) Antiangiogenic at 5 μg per pellet in chorioallantoic membrane assay[188]
7	Ag_2O or *Trametes versicolor* laccase	**7d**	Angiogenesis	In ECV304 cells proangiogenic: Cyclin D1 and VEGF↑ at 0.1-10 μg/mL[191]; or Antiangiogenic: •NO ↓, VEGF↓ at 20-80 μM[192]	Antiangiogenic on aortic endothelial cells, and VEGF ↓ in swine granulosa cells, both at 1 μM[190]
8	AIBN in ethyl linoleate / ACN[193]	**8g**	NO production of LPS-activated BV-2 cells	IC_{50} = 87.62 μM[196]	IC_{50} = 51.80 μM[196]
		8g	Adipocyte differentiation, fat accumulation	Inhibition at IC_{50} > 100 nM[197]	Inhibition at IC_{50} < 10 nM[197]
9	Tyrosinase, with or without the presence of GSH	**9q**	Human placenta GST π	Inactive at 25 μM, 90% inh. at 100 μM; mixed mode inh. (K_i = 53 μM, K_i' = 97 μM)[b] [199]	85%-90% Inh. at 10-20 μM; irreversible mixed mode inh. (K_i = 20 nM, K_i' = 50 nM)[199]
		9r			88%-95% Inh. at 5-25 μM; reversible competitive inh. (K_i = 0.74 μM)[199]

[a]Estimated values.

[b]GST π inhibition was determined by the CDNB method; data in the table refer to results obtained with respect to GSH, K_i and K_i': inhibition constants for the inhibitor-enzyme and inhibitor-(enzyme-substrate complex) bindings, respectively.

For example, vitamin C was found to exert an antagonistic effect on the cytotoxic activity of several antineoplastic drugs, and the antagonism was much stronger than that expected from the weak decrease in ROS levels.[136] On the other hand, large doses of vitamin C were recently found to selectively kill *KRAS* or *BRAF* mutant colorectal cancer cells, and this activity was rather connected to DHA, not ascorbic acid.[137-139] This is an important finding since *KRAS* and *BRAF* are major oncoproteins in the gastrointestinal tract, and their mutation is closely connected with the progression and therapy resistance of colon cancer.[140] Whether or not, and if yes, to what extent IKK inhibition by DHA plays a role in the above, remains unknown. Another interesting example to the possible pharmacological importance of DHA is the antiviral activity of vitamin C. DHA was reported to have a much stronger antiviral activity as compared to that of ascorbic acid against Herpes simplex virus 1 (HSV-1).[141] Based on the weaker cytotoxicity observed with DHA than with ascorbic acid, Furuya et al concluded that the antiviral effect of DHA is unlikely to arise due to an effect on the host cells.[141] However, HSV-1 replication is highly NF-κB dependent and IKK inhibition can greatly reduce virus yield through inhibition at the transcription level,[142,143] exactly as found in the above-mentioned study.[141] As an important consequence of this possible mechanism of action for the antiviral activity of DHA, one could conclude that IKK inhibition by DHA matters enough to make relevant the pharmacological differences between the two forms of vitamin C. In other words, it would mean that oxidative stress directly modulates the specific bioactivity of this antioxidant through its metabolite.

Another good example for the biological relevance of a compound emerging from RONS scavenging by an antioxidant is the Prokai cycle of estrone and estradiol that explains their neuroprotective activity. Prokai et al found that •OH radical scavenging by estrone yields a *p*-quinol A-ring-containing intermediate that subsequently undergoes a NADP-dependent enzymatic reduction back to estrone, without contributing to oxidative stress on its own but becoming able to scavenge further radicals.[144-146] *In vivo* administration of the analogous *p*-quinol derivative of estradiol (DHED) demonstrated no estrogenic activity, and it was rapidly taken up into the brain where it initiated the above-mentioned redox cycle; therefore it acted as a targeted neuroprotective agent without any apparent systemic side-effects.[147-150] On one hand, this provides an attractive strategy to deliver estrogens to the brain through their pro-drugs. On the other hand, it also represents a good example for a phenolic antioxidant (estradiol or estrone) to be transformed to a metabolite (eg DHED) on a free radical scavenging-related manner. Certainly, such a nonaromatic metabolite will have a significantly different bioactivity profile than its parent compound (ie no estrogenic effect in this case; other specific bioactivities were not studied). The reaction mechanism for the above-mentioned redox cycle is shown in Figure 6.

Concerning possible chemical approaches to explore the RONS scavenging-related metabolic fingerprint of antioxidants, biomimetic oxidative chemistry offers a readily available toolkit. Development and application of biomimetic or bio-inspired chemical reactions represent a rapidly emerging approach in today's chemistry. Such reactions use transition metals as catalysts either in free forms or in their complexes, for example as metalloporphyrins,[151] to perform various oxidative transformations similar to those observed in biological systems. A common feature of these types of reactions is that the oxidizing agent is an activated (ie reactive) oxygen or nitrogen species formed by electron transfer from the transition metal, aiming to mimic the way oxidase enzymes work.[152,153]

Accordingly, it is unsurprising that biomimetic approaches are extensively used in the total synthesis of natural products,[154] including various functionalization or cross-coupling reactions of phenolic/polyphenolic compounds.[155-157]

Nevertheless, more than providing a remarkable toolkit for solving synthetic chemical challenges, biomimetic oxidative chemistry also offers a platform to study the oxidative metabolism of drugs.[158] Such approaches require the best possible models of selected metabolizing oxidase enzymes. From a different perspective, however, biomimetic oxidative reactions can also be considered as reasonable chemical models for studying the potential impact of RONS on compounds including for example dietary antioxidants. Apparently, no related systematic studies have been performed so far, but sporadic reports can be found where one or more bioactivities of an

antioxidant were directly compared in the same pharmacological model to those of its oxidized metabolites obtained by biomimetic oxidative chemistry.

In the following sub-sections, chemically oxidized derivatives of compounds **1**-**9** will be presented from this perspective. For each compound, selected examples are discussed, in which the bioactivity of the parent antioxidant on specific pharmacological models could directly be compared to that of its chemically stable metabolite(s). An overview on these comparisons is provided in Table 1.

Since the field is extremely rich in reports on antioxidant natural products, it is almost certain that the below examples do not provide a complete coverage. Still, their chemical and pharmacological diversity might provide an overview suggesting that oxidative stress-related metabolites may represent an important piece of the antioxidant puzzle, which is frequently overlooked.

4.1 | Curcuminoids

In the case of curcumin (**1**; Figure 1) and its derivatives, oxidative changes have been extensively studied over the years as a likely explanation for the polypharmacology of these compounds.[56,159] Spontaneous aerobic oxidation of curcumin leads to the formation of a bicyclopentadione derivative (**1u**) as a major product[160]; for its chemical structure, see Figure 1B. A most recent, in-depth mechanistic study identified several intermediates (eg **1t** and **1v**) and by-products (**1s**, **1w**, **1x** and **1y**) of this metabolite, arising from a course of multiple chemical rearrangements initiated by the phenoxyl radical.[161] This radical is obviously among the primary products when curcumin participates in ROS scavenging. The broad chemical diversity of the subsequently forming intermediates and end-products raises many exciting questions concerning their bioactivities in relation to their parent compound **1**. Unfortunately, as of today, little is known about them. Topoisomerase II poisoning activity of curcumin [162,163] is clearly associated with its transformation: its activity could be observed only in oxidative conditions.[164] Moreover, the same oxidative activation applies for poisoning topoisomerase IIα by demethoxycurcumin (**2**) and bisdemethoxycurcumin (**3**): the activity of both curcuminoids significantly increased when combined with $K_3Fe(CN)_6$, a biomimetic oxidative reagent.[165] Transformation route of both **2** and **3** was found to be related to that of curcumin, and **2** also yielded a bicyclopentadione derivative (**2r**) as major metabolite. Both **2** and **3** were, however, more stable to resist autoxidation, and **3** even required the addition of $K_3Fe(CN)_6$ or H_2O_2 and horseradish peroxidase (forming ROS) to transform to a bisdemetoxy-spiroepoxide (**3k**), and stop at that phase without converting to a bicyclopentadione.[165] In view of most recent studies, however, it seems that a great deal of the bioactivity of curcuminoids is connected rather to the intermediates of the autoxidation and not the stable bicyclopentadiones.[166,167] Sanidad et al found that, while curcumin (**1**) had potent antiproliferative and proapoptotic activity against colon cancer cells, and exerted anti-inflammatory activity through inhibition of the NF-κB signaling, both the mixture of the degradation products and **1r** were weaker or rather inactive in these bioassays.[166] However, by using an experimental setup that allowed *in situ* formation of the autoxidized products, Edwards et al found that NF-κB inhibition by curcumin depends on oxidative activation, and that this is due to the reactive electrophile intermediates covalently binding to signaling proteins, such as for example IKKβ. Very interestingly, and in line with the perspective discussed here, they could clearly connect this activity to the oxidative status of the cells, in other words, they demonstrated that oxidative stress modulates curcumin's bioactivity through the *in situ* formation of oxidized intermediates.[167]

Curcumin is also known to rapidly decompose in cell culture medium under aerobic, physiological conditions, that yield a set of cleaved products, such as trans-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5-hexenal (**1z**) as major product, and ferulic acid (**1za**), ferulic aldehyde (**1zb**), feruloyl methane (**1zc**), vanillic acid (**1zd**), and vanillin (**1ze**) as minor metabolites.[168,169] These compounds have received attention in numerous studies with curcumin, leading to an intense discussion on their potential role in some previous controversial findings.[159] For example, they were suggested as potentially responsible for the xanthine oxidase (XO) inhibition by curcumin,[170] which was previously reported both with positive [171] and negative [172] results. Based on *in silico* predictions validated by experimental XO inhibitory activities of quercetin and luteolin, the side-chain cleaved metabolite **1z** was suggested as a strong inhibitor of the enzyme, while curcumin itself was too large to bind to the active center of XO.[170] In

contrast to this, the testing of curcumin and its cleaved metabolites **1z**, **1za**, **1zb**, **1zd**, and **1ze** against human recombinant cytochrome P (CYP) enzymes CYP1A2, CYP3A4, CYP2D6, CYP2C9 and CYP2B6 showed that while curcumin (**1**) can inhibit all of them, none of the metabolites were significantly active in this regard.[169]

In a recent debate initiated by comments on the results of Dhillon et al on the potential therapeutic use of large doses of orally administered curcumin in cancer patients,[173] the same degradation products were hypothesized to be responsible for its beneficial effect.[174] In their response, the authors disapproved this theory and presented evidence for the inactivity of **1za** and **1zc** for suppressing the activation of NF-κB, in contrast with the case of curcumin.[175] While the clinical relevance of the above-mentioned recent findings by Edwards et al[167] on this pathway is unclear, one could hypothesize a similar oxidative stress-related formation of reactive intermediates.

The theory suggesting high importance of the cleaved metabolites (**1z**-**1zc**) in the bioactivity of curcumin [159] has also been challenged; based on the fact that under biologically more relevant conditions they form in smaller amounts than the bicyclopentadione compound **1u**.[176] However, when encapsulated curcumin was administered to healthy human subjects through enriched bread, to at least partly protect it from phase I and II metabolism, vanillic and ferulic acids (**1zd** and **1za**, respectively) were detected as major metabolites.[177] While it is unknown how this would correlate to the amount of **1u**, which should possibly form *in vivo* depending on the oxidative status, these cleaved products may also play their role in curcumin's bioactivities, particularly concerning the XO and CYP enzyme inhibition.[178]

Altogether, curcumin (**1**) represents an excellent example for an antioxidant that can undergo many different types of oxidative transformations, apparently leading to an oxidative status-related change in its bioactivity profile. Considering that much of the related dispute appears to originate from different applied oxidative conditions, one must assume that the metabolite pattern arising in a biological system will greatly depend on the local microenvironment, for the types and levels of RONS to be scavenged.

4.2 | Resveratrol

In view of the possible biological role of oxidized metabolites of resveratrol (**4**; Figure 2), its bioactivity on lipoxygenase (LOX) enzyme demonstrates an interesting example. Resveratrol was reported as a potent competitive inhibitor (IC_{50} = 13 μM) of the dioxygenase but not the hydroperoxidase activity of LOX. At the same time, LOX gradually oxidized resveratrol to an *in situ*, non-characterized derivative that was suggested to be similarly active on the dioxygenase activity of LOX as resveratrol itself.[179] More recently, resveratrol was involved in a study where 30 polyphenols were subjected to an Fe^{3+} catalyzed biomimetic aerobic oxidation and the resulting mixtures were screened for specific LOX inhibitory activity through the xylenol orange (FOX) assay that measures the hydroperoxidase activity.[180] As expected, resveratrol itself was inactive at a concentration as high as 100 μM, while its oxidized mixture exerted 98% inhibition at a concentration equivalent to 50 μM of resveratrol, despite the rather low (33%) conversion. Isolation of the predominant metabolites led to the identification of two active dihydrobenzofurane dimers, compound **4m** and δ-viniferin (**4n**); for the relevant structures, see Figure 2B. Further investigations revealed that the metabolite profile is highly dependent on the reaction conditions: by using excess $CuCl_2$ in ethanol, compound **4m** was obtained at relatively higher yields, while oxidation with DPPH radical made compounds **4n** and **4o** (the latter inactive as LOX inhibitor) as preferred metabolites.[180] Besides the low conversion of resveratrol (**4**), it should be highlighted that more than 20 products were visible on the HPLC fingerprint of the active mixture. This mixture exerted a complete inhibition of LOX at a concentration equivalent to 50 μM of resveratrol (**4**), implying that the activities reported for the individual metabolites **4n** and **4m** (IC_{50} values ca. 17 and 62 μM, respectively) cannot explain the activity observed for the mixture that contained these compounds in a few percent amount only.[180] This suggests either the presence of further active metabolites exerting stronger LOX inhibitory activities than compounds **4m** and **4n**, or strong synergistic interactions between the minor compounds of the reaction mixture.

Considering the very low (free) resveratrol concentrations achievable *in vivo* (see Section 3.2), the biological relevance for the RONS-dependent formation of such dimers and/or oligomers might seem to be at least questionable. Certainly, it seems there can be only a very low chance that the intermediates formed upon RONS

scavenging would react with each other instead of for example GSH or other, endogenous compounds present in excess amounts. This is, however, not necessarily the case. NMR studies [181] and high-level *in silico* calculations [182] provided evidence for a very strong self-association of stilbenes stabilized by π-π interactions in aqueous environment, which could explain the inconsistencies in the regio- and stereoselectivity observed in oxidative coupling reactions similar to the case above.[182] In addition to these implications, this also means that fully π-conjugated stilbenes (eg resveratrol) preferably stick together in the aqueous environment provided by a living organism. As such, there might be a reasonably high chance for the formation of dimer/oligomer metabolites of such antioxidants through cross-coupling upon RONS scavenging, and for related modulation of their bioactivity.

4.3 | Hydroxycinnamates

The oxidized metabolites of hydroxycinnamates may provide interesting and valuable insights into the antitumor activity of their parent compounds.

In a most recent, proof-of-concept study of our research group, methyl-*p*-coumarate (**5**; Figure 3) was studied for its potential to form bioactive metabolites upon free radical scavenging.[183] A hypervalent iodine reagent (PIFA) was used to obtain oxidized metabolite mixtures of compound **5**. PIFA can oxidize phenolic compounds through single-electron transfer (SET) whose intermediate can transform into the same phenoxyl radical upon deprotonation as that forming in a hydrogen-atom transfer (HAT) reaction. Therefore, it was expected that the use of this reagent would result in metabolites like those forming through free radical scavenging. Two metabolites (**5e** and **5f**; see Figure 3B) were identified with antitumor activities much stronger than that of compound **5**. Graviquinone (**5e**) had over 2 orders of magnitude stronger cytotoxicity on certain resistant cancer cell lines as compared to compound **5**. Ten μM of graviquinone (**5e**) induced DNA damage in NCI-H460 and NCI-H661 lung carcinoma cells while it exerted DNA protective activity in normal HaCaT cells as determined by the relative expression of Histone 2A.X. At the same concentration it modulated DNA damage response through the inhibition of Checkpoint kinase-1 (Chk1) phosphorylation and the induction of Checkpoint kinase-2 (Chk2) phosphorylation in MCF-7 cells. It was also demonstrated that graviquinone (**5e**) can be formed as the result of the Fenton reaction of compound **5**. *In silico* studies suggested that **5e** is a kinetic metabolite when methyl-*p*-coumarate (**5**) scavenges $^{\bullet}$OH radicals. The fact that cytotoxic activity of methyl-*p*-coumarate (**5**) was also potentiated by H_2O_2 is indirect evidence for the oxidative stress-related *in situ* formation of antitumor metabolites; *for example* compound **5e**.[183] It is also worth noting the structural similarity of compound **5e** to the above-mentioned *p*-quinol estrone derivative DHED (see Figure 6). This suggests that *p*-phenols in general can be transformed into their *p*-quinol analogs upon free radical scavenging. Moreover, such *p*-quinol derivatives, including DHED itself, might be worthy of further study for activity on checkpoint kinases Chk1/Chk2, two of the key enzymes involved in response to DNA damage. These kinases are important in cancer development and, possibly, treatment.[184]

Concerning other hydroxycinnamates, methyl caffeate (**6**) was reported as a weak-moderate antitumor agent against various cell lines with *in vitro* antiproliferative IC_{50} values at the medium-low micromolar range against MCF-7 breast cancer cell line (ca. 110 μM).[185] Still, there are some studies showing much stronger antitumor effects for this compound, for example Balachandran et al observed cytotoxic activity of **6** against MCF-7 cells with an IC_{50} value as low as 0.62 μM, and the involvement of several pro- and antiapoptotic genes in apoptotic cell death.[186]

Ag_2O catalyzed biomimetic oxidation of a set of cinnamic acid methyl esters including compound **6** yield metabolites with highly potent antitumor activity, and compound **6m**, an oxidized dimer of **6**, exerts a particularly strong antiproliferative activity (IC_{50} < 10 nM) against breast cancer cell lines MDA-MB-435, MDA-N and BT-549, and sub-micromolar activity against several other cell lines. Moreover, the 2*R*,3*R* enantiomer of compound **6l** (but not the inactive 2*S*,3*S*) was identified as an antitubulin agent as strong (IC_{50} = 6.0 μM) as combretastatin A-4,[187] a well-known natural inhibitor of tubulin polymerization. The same compound, the 2*R*,3*R* enantiomer of **6l**, was also found to exert potent antiangiogenic activity apparently without interfering with fibroblast growth factor-2 (FGF-2) or vascular endothelial growth factor (VEGF).[188] Moreover, in a recent, in-depth pharmacological study using *in vivo* and

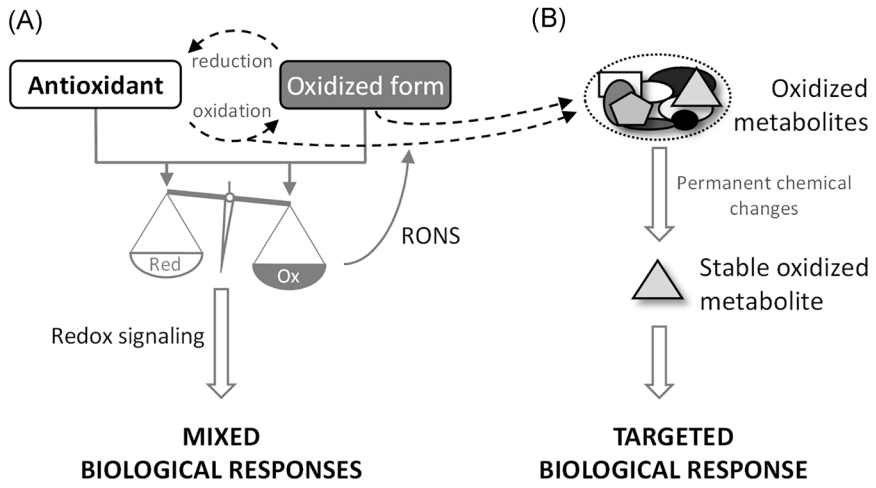


FIGURE 7 Contextualization of the chemical perspectives discussed in Section 4, in connection with the canonic view on antioxidant activity. (**A**) Role of antioxidants in interfering with the redox balance and redox-modulated biochemical pathways. The antioxidant may enter a redox cycle and exert its bioactivities through both its reduced and oxidized form. (**B**) Potential importance of minor, chemically stable oxidized metabolites that may form due to free radical scavenging. Depending on the antioxidant's chemical properties, the type of ROS/RNS scavenged, re-arrangements of emerging reactive intermediates and/or other secondary reactions, etc., a complex mixture of oxidized metabolites is expected to be present and interact with various cellular mechanisms involved in the regulation of many biochemical processes. This localized metabolite pattern may be characteristic to the microenvironment, implying a crosstalk between redox signaling and a co-existent "antioxidant-metabolite signaling". Since individual oxidized metabolites tend to show higher chemical complexity than their parent antioxidant, they are also expectable to act in a more targeted way

3D cell culture models, Yin et al found that compound **6l** has a significant potential for further development of a clinically applicable antimetastatic agent.[189] Of significant pharmacological interest, compound **6l** exerts its activity mainly on the microenvironment of the tumor through inducing the IL-25 secretion of tumor-associated fibroblasts, and this effect could be achieved in mice at doses as low as 20 to 100 $\mu g\, kg^{-1}$ body weight.[189] The activity of compound **6** was not investigated, and it is hard to imagine that its activity would be like that of its oxidized metabolite **6l**. As for the antiangiogenic effect, compound **6** was reported to be inactive in zebrafish.[190]

In another related study, the racemic mixture of compound **7d**, obtained enzymatically from methyl ferulate (**7**), exerted a similarly strong *in vitro* antiangiogenic effect to that of **6l** (2*R*,3*R*). A significant decrease in the VEGF production was found in swine granulosa cells at 1 μM concentration of this compound, and it also retained the antioxidant activity of its parent compound, methyl ferulate (**7**).[191] Contradictory results have been reported with ferulic acid and its effects on angiogenesis.[192,193] Ferulic acid was found to up-regulate cyclin D1 and VEGF in endothelial cells, leading to a proangiogenic effect,[192] while in the other study, antiangiogenic effect was found through downregulation of •NO, which, depending on the cell type, can lead to VEGF downregulation.[193] This contradiction may be connected to the perspective presented here: namely, results that depend on the oxidative status of the studied system. The effect of ferulic acid would be tuned in either direction through possible variations in its metabolite profile likely including compounds similar to those obtained from methyl ferulate.

4.4 | Secoisolariciresinol

The oxidation of secoisolariciresinol (**8**; Figure 4) was previously studied by employing 2,2′-azobis(isobutyronitrile) (AIBN), a radical oxidant initiator and ethyl linoleate dissolved in acetonitrile producing a lipophilic environment

that models biological membranes. Several products were obtained (for structures, see Figure 4B) including a quinone methide (**8f**), peroxy-*p*-quinol adducts with either the lipid or a thermally decomposed product of AIBN (**8e** and **8d**, respectively), and lariciresinol (**8g**), another natural lignan derivative.[194] Among these products, bioactivity data are only available for **8g**. This compound is also a potent antioxidant, although slightly weaker than its parent compound **8**.[195] Compounds **8g** and **8** were also isolated from the trunk of *Berberis koreana*, and both of them studied for their capacity to inhibit •NO stress produced in LPS-activated BV-2 microglia cells. While lariciresinol (**8g**) was found somewhat more active than secoisolariciresinol (**8**), with IC_{50} values of 51.8 and 87.6 μM, respectively, this is a minor difference with little if any relevance in terms of a RONS-scavenging related bioactivity change.[196] However, much larger differences in the inhibitory activities of **8g** and **8** can be observed on differentiation and fat accumulation of adipocytes (IC_{50} < 10 nM for **8g** vs. IC_{50} > 100 nM for **8**).[197] These 1 to 2 orders of magnitude difference in potency means that even small amounts of **8g** formed from **8** may significantly modulate the overall bioactivity, perhaps in an oxidative stress-related manner. Nevertheless, **8g** formed through the oxidation of **8**, can still be metabolized to the same end-products as its parent compound. If **8g** reaches the large intestines, microbiota can transform it first to **8**, and subsequently to the bioactive metabolites enterodiol (**8c**) and enterolactone (**8d**) (Figure 4A and 4B).

4.5 | Luteolin

An obvious structural change of luteolin (**9**; Figure 5) upon RONS scavenging is the reversible formation of *o*-quinone from the catechol group - a change that is also catalyzed by many oxidase enzymes. This *o*-quinone can than either enter a redox cycle where it can contribute to the formation of certain types of ROS or react as a Michael acceptor with an appropriate nucleophile, such as glutathione (GSH). Due to their relatively high reactivity, such quinones are usually considered as toxic intermediates, while they can also have an effect on cellular signaling targets including the IKK complex, Keap1, and the JNK pathway through the Glutathione S-Transferase π 1 (GST π1). For more insight into this subject, see the recent perspective by Bolton and Dunlap.[198]

In connection with the above-mentioned reaction of luteolin quinone with GSH, an interesting study on its specific bioactivity was published by Balyan et al, that compared the GST π inhibitory activity of luteolin (**9**), its *o*-quinone (**9q**), and its glutathione conjugate (**9r**) [199] (for the structures, see Figure 5B). Luteolin itself exerted a weak or rather negligible GST inhibition, while both the quinone (**9q**) and the glutathione conjugate (**9r**) were identified as strong inhibitors. Luteolin (**9**) was a weak, reversible noncompetitive inhibitor. The quinone (**9q**) inhibited GST in an irreversible manner through a mixed mechanism. The glutathione conjugate (**9r**) acted as a strong, reversible competitive inhibitor of the GST enzyme.[199] Such a mechanism may play an interesting role in the antitumor effects of other catechol antioxidants as well, particularly in view of the typically elevated ROS levels in cancer cells [200,201] and the importance of various GST isozymes in detoxification of the cell and, consequently, in chemoresistance.[202-204]

5 | OUTLOOK

Dietary antioxidants interfere with biological processes through several mechanisms of action, including free radical scavenging, modulation of redox signaling pathways and transcription factors, and the formation of sometimes very complex metabolite fingerprints. All these mechanisms are clearly interconnected at multiple levels.

It is of particular interest that the formation of oxidized metabolites may occur through scavenging RONS, and, accordingly, this may directly depend on the level of oxidative stress. As herein discussed, a wide variety of pharmacologically active metabolites may form through the interaction of an antioxidant and a reactive oxygen or nitrogen species. Interestingly and somewhat unsurprisingly, such chemical transformations usually result in more complex chemical structures as compared to their parent compounds. In contrast, metabolic transformations by gut microbiota, for example, point towards simplicity, for example reduction, fragmentation – see a comparison between

Figures 1-5A and Figures 1-5B. Such increases in structural complexity imply a possible increase in target specificity. Since the oxidized metabolite pattern is dependent on the oxidative status, it must therefore strongly depend on the microenvironment, and so must be the related bioactivity pattern. In connection with the rapidly emerging "omics" concepts, this could possibly be translated as an existence of some kind of a "scavengome" for these antioxidants, defined as the chemical space represented by all their possible RONS scavenging-related metabolites influencing the overall bioactivity observed in complex biological systems.

From a biological point of view, specific bioactivity patterns of this "scavengome" would imply an oxidative stress-related, "direct" signaling role of locally emerging antioxidant metabolites. This may provide new answers and possible alternative explanations to much of the controversy still present in the field.

From a pharmacological and drug discovery perspective, the oxidative modulation of antioxidants' bioactivity suggests that such compounds may at least partly function as oxidative stress-activated pro-drugs. Considering that only marginal attention has been assigned to the RONS scavenging-related structural changes carried by antioxidants and to the related pharmacological consequences, these point towards an unexplored segment of chemical space that is apparently a treasury of bioactive molecules. Higher target specificity of these metabolites as compared to their parent antioxidants may also make them more attractive as lead compounds for drug discovery. A contextualization of this concept with the generally accepted mechanisms of function of antioxidants is presented in Figure 7.

From a practical point of view, it should be noted that any research efforts attempting to explore this "scavengome" would extensively rely on *in silico*, as well as modern analytical and preparative chromatographic techniques. A drug discovery initiative aiming to successfully explore the pharmacological potential of such metabolites should use state-of-the-art metabolomic techniques with oxidized mixtures obtained by various conditions. Moreover, considering the typically low expectable yield of individual oxidized products identified as hits on a given target, large-scale preparations are necessary for further development and these would also require precise, sophisticated methods. The rapidly developing field of oxidative continuous flow chemistry[205] may provide the required flexibility and timely throughput to overcome challenges for yield optimization.

Finally, it is important to underline that the oxidative stress-induced alteration of the bioactivity pattern of a given antioxidant is not necessarily beneficial for the organism affected by it. This may lead to undesirable side-effects, such as toxicity, or a loss of its *in vitro* promising activity to the *in vivo* and/or clinical phase studies (see some of the examples in Section 3). Even though few antioxidants have reached the bedside, most of the initially promising drug candidates yielded disappointing clinical outcomes.[206] Considering the environmental-related emergence of chronic diseases where oxidative stress plays a central role, there is greater need for effective and safe antioxidants with therapeutic relevance. Pharmacological aspects of the oxidative status-related metabolic fingerprint of an antioxidant may not only provide new candidates to develop on their own right, but also lead to understanding what chemical design is necessary to prevent certain unwanted modifications whenever that antioxidant meets its nemesis, a reactive oxygen or nitrogen species.

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